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Request for Grant of a Patent

The Patent Office
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1. Your reference — P79610

2. Patent Application number *(the Patent Office will fill in this part)* — 9907962.6

08 APR 1999

3. Full name, address and postcode of the or of each Applicant *(underline all surnames)*

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Patent Office ADP Number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation — 6172290003

4. Title of the Invention — Monitoring of liquids for disease-associated materials

5. Name of your Agent *(if you have one)* — URQUHART-DYKES & LORD

"Address for Service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Patents ADP Number *(if you know it)* — 16044005

6. If you are declaring priority from one or more earlier Patent Applications, give the country and the date of filing of the or of each of these earlier Applications and *(if you know it)* the or each

Country	*Priority application No. (if you know it)*	*Date of Filing (Day/month/year)*

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Number of earlier application	*Date of Filing (Day/month/year)*

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a) any Applicant named in part 3 is not an inventor, or
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Request for Substantive Examination *(Patents Form 10/77)*	0
Any other documents *(please specify)*	0

11. I/We request the grant of a Patent on the basis of this Application

Signature Date

Urquhart-Dykes & Lord

URQUHART-DYKES & LORD 8 April 1999

12. Name and daytime telephone number of person to contact in the United Kingdom

Mr. H.W. Austin - 01792 474327

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Monitoring of Liquids for Disease-Associated Materials

The present invention relates to the monitoring of liquids for disease-associated materials and more specifically to the monitoring of liquids for materials associated with autoimmune and other diseases, all using non-invasive means.

At present, the principal methods for monitoring infectious, and autoimmune disorders, cancer and the like, such as Alzheimer's disease, multiple sclerosis, spongiform encephalopathies etc. are invasive techniques involving the monitoring of pathological changes in surgically accessible tissue. Similarly, principal methods for monitoring various cancers also involve invasive techniques. Amyloid plaques, for example, are a common neuropathological feature of Alzheimer's disease and would conventionally require invasive surgery in order to be detected, which is generally undesirable. These surgical methods are expensive and time consuming and are often only undertaken when a disease is at an advanced stage. Therefore, an accurate, non-invasive test would provide means to aid in the early detection and diagnosis of various disorders, thereby improving the possibility for the early treatment of the disease, hence potentially increasing the chances of combatting or arresting the disorder.

My own European specification 0854364 discloses a method for the diagnosis of neuro-degenerative disorders in an animal using a sample of body fluid from the animal, which method comprises:

(a) concentrating a protein associated with a neuro-degenerative disorder in the sample, the concentration being by contacting the sample with a solid, non-buoyant particulate material having free ionic valencies (such as calcium phosphate in granular form); and

(b) monitoring the resulting protein associated with a neuro-degenerative disorder concentrated on the particulate material.

There is no suggestion that the method could be used for infectious diseases or diseases such as cancer.

It is an aim of the present invention to provide a method for monitoring liquids for disease-associated materials, which can be used for detection of materials associated with diseases such as cancer, as well as neuro-degenerative disorders.

It is a further aim of the present invention to provide non-invasive means for the detection of various materials associated with cancer, and autoimmune and other disorders. It is a further aim of the present invention to provide means for the detection of materials associated with cancer and autoimmune and other disorders at an earlier stage than is possible using techniques currently available (particularly where the etiology is unknown or difficult to determine).

According to a first aspect of the present invention, there is provided a method of monitoring liquids for the presence of disease-modified or associated proteins, comprising the steps of:

(i) providing a sample of said liquid;

(ii) passing said sample through a solid filter medium having free ionic valencies so as to complex at least one disease-modified or associated protein or a fragment thereof present in said sample and/or at

least one virus or a fragment thereof present in said sample to said medium; and

(iii) monitoring at least a part of said complexed disease-modified or associated protein or fragment thereof and/or complexed virus or fragment thereof, wherein the presence of at least a part of said disease-modified or associated protein and/or at least a part of said virus is indicative of an association of said liquid with the relevant disease.

The relevant disease may, for example, be an autoimmune or similar disorder.

It is a preferred feature of the present invention that the sample of liquid body fluid comprises a urine sample or a sample of another body fluid (such as serum or cerebral spinal fluid) comprising detectable levels of a disease-modified protein or detectable levels of viral matter.

According to the present invention, the filter medium preferably comprises a sheet-like member with a pore size ranging from 1 to 100 microns. The pore size of the filter may be varied according to the size of the particles to be entrapped. Furthermore, the filter preferably comprises a gauze and/or cotton fibre.

According to the present invention, the disease-modified protein is a protein or a fragment thereof which is modified due to a disease in a host body and which protein or fragment thereof is excreted as the disease process begins. For example, it is known that amyloid β-protein is derived from amyloid β-precursor protein which is encoded by a normal host gene mapped to chromosome 21. In Alzheimer's disease, amyloid β-precursor protein slices into 3 segments as the disease

progresses, one of the segments, typically the middle segment, being amyloid β-protein (a 4KDa protein which forms plaques as seen in brain sections of Alzheimer's patients). The remaining two segments of the amyloid precursor protein have not been demonstrated in brain tissue of Alzheimer's patients. In patients testing positive for Alzheimer's disease, the presence of C-terminal segments of the amyloid β-precursor protein may be shown. In contrast, the urine of patients testing negative for Alzheimer's disease will not contain segments of the amyloid β-precursor protein. Such protein modifications have been found to occur in both infectious and non-infectious diseases, such as cancer.

According to the present invention, when the disorder is Alzheimer's disease, the disease-modified protein is typically amyloid β-protein. Furthermore, when the disorder is multiple sclerosis, the disease-modified protein is typically myelin. When the disorder is a bovine spongiform encephalopathy or Creutzfeldt Jakob disease, the disease-modified protein is typically protease-resistant protein.

According to the present invention, viruses such as cytomegalovirus, papillomavirus or the AIDS virus excreted in urine may be detectable.

According to a further preferred feature of the present invention, the disorder may be Alzheimer's disease, multiple sclerosis or a spongiform encephalopathy. Furthermore, since disease modified proteins have been demonstrated in cancer, for example in cancer of the cervix, the method according to the present invention may also be applicable to the detection and subsequent diagnosis of various forms of cancer. Similarly, various viruses associated with certain cancers, growths etc. have also been demonstrated in urine samples.

In an alternative embodiment of the present invention, non-buoyant particulate material having free ionic valencies may be used instead of, or in addition to, the filter medium. Such particulate material, typically comprising calcium phosphate, a hydroxyapatite or the like, may be used in a method for monitoring for the presence of proteins associated with cancer, infectious diseases or the like.

Hydroxyapatite and similar media, as ion-exchange chromatography media, have previously been used to purify and concentrate viruses and their related soluble antigens. Such media have had limited application in the clinical diagnosis of human and animal diseases largely due to the impracticality of handling large sample volumes with slow transit of liquids through an ion-exchange column. A further problem is the low concentration of disease-related proteins which are in competition with contaminating proteins for exchange sites on the particulate materials. The method according to the present invention overcomes some of these difficulties by use of a medium which discriminates adsorption of albumin (in other words, proteins such as albumin are selectively not complexed as the medium is caused to lose the charge that allows albumin to complex).

According to the present invention, following concentration of viral or protein matter on the filter, detection of the viral particles on the filter surface may be carried out using an electron microscope. Furthermore, additionally or alternatively, detection of proteins using western blotting techniques may be carried out following concentration.

The present invention has been described with particular reference to purification and detection of protein and viral matter from urine samples. According to a further

embodiment of the present invention, the filter technology may be used to purify viral samples from water. The method according to the invention may prove useful in the detection of viral and/or bacterial matter from sea water, swimming pool water, tap water or the like.

The present invention will now be described in more detail with reference to the following examples.

Methods and materials

One hundred ml, or larger, urine specimens, were collected in 50ml tubes, three times, from 10 clinically diagnosed Alzheimer's patients and 10 healthy individuals of similar age group and sent fresh to the laboratory. After centrifugation at 1000g for 10 minutes to remove gross debris, the supernatant was transferred to fresh 50ml polypropylene centrifuge tubes. One 50ml aliquot of the specimens was used and the rest frozen. To each tube, 1ml buffer was added, mixed and then 500μl non-buoyant particulate flock added. Tubes were left on a roller for 30 minutes at room temperature and agitated every 10 minutes. The tubes were then centrifuged at 200g for 3 minutes and the pellet collected and supernatant discarded. The pellet of non-buoyant particulate flock with protein fragments adsorbed was transferred to a microfuge tube and suspended with another 1ml buffer and centrifuged. This step was repeated twice. Following concentration of the urine, buffer was removed by centrifugation at 10,000g for 1 minute and 250μl sample buffer (3X) was added, mixed and followed by boiling for 3 minutes. The supernatant was collected into a fresh tube after centrifugation at 10,000g for 1 minute. This sequence provides an approximate concentration of 200 times.

Western Blotting

After boiling, the samples were run on sodium dodecyl

sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels. For each run, 20μl of the 250μl of the concentrate was loaded. Electrophoresis was carried out using 10% polyacrylamide gel using BIO-Rad mini-gel apparatus. Secretory amyloid precursor protein C-terminal was used for the control. After the run, the proteins were transferred to PVDF membrane. Unadsorbed sites were then blocked using milk blocking buffer with sod-azide. A first amyloid precursor protein antibody 369 was made up in blocking buffer which was left to incubate for one and a half hours. The membrane was then washed three times in wash buffer. A second antibody, conjugated to a marker enzyme, (which was also made up in secondary blocking buffer without sod-azide) was left to incubate for one and a half hours and then washed three times in wash buffer without sod-azide. Developing: 1 part of A+ 1 part of B on membrane for 1 minute. The liquid was blotted and the membrane exposed for 30 seconds and 5 minutes and the film developed.

Results

Western immunoblots prepared from urine concentrates of all Alzheimer's patients showed positive reactivity to the antibody raised to the amyloid precursor protein segments. Samples include collection and processing on different days from the same patients. Apart from quantitative differences, in most cases, two bands of 27 to 30 KD and 7 KD were seen. In some patients, there was a third band, just below the 27 to 30 KDa band. None of these bands were seen in one patient with Parkinson's disease also included in this study. No bands were seen in control cases. For comparative purposes, urine specimens from some Alzheimer's disease cases were run in SDS-PAGE gel without concentration. None of the bands were seen in SDS-PAGE gel in these runs.

Purification of Viral Samples from Water

Water samples were collected from laboratory tap and also from the River Tyne in gallon containers. A 2 to 5% suspension of faeces which contained rotavirus was prepared in PBS. One ml of the suspension was added into one gallon water sample, mixed by shaking for 2-3 minutes. To each container, 10ml buffer was added, mixed and then the cap of the container was replaced with a ion-exchange filter. The liquid was poured by gently tilting the container and was discarded. The filter paper was removed and immersed in 250μl saturated versene. Following the concentration 50μl of versene was used to prepare the grids by low speed centrifugation technique (Narang *et al*, 1980, Lancet, i, 1192-1193). The grids were stained with PTA and examined with an electron microscope. Rotavirus was found in all water samples with added faecal suspension, both in the tap and river concentrated by filter method. The filter method can be used to concentrate virus from river, sea and swimming pools water. The number of virus particles seen by an electron microscope demonstrated that the concentrated water samples could be used for analysis by Western Blotting.